Exhibit 84

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Potentiation of Endogenous Fibrinolysis and Rescue from Lung Ischemia/Reperfusion Injury in Interleukin (IL)-10-reconstituted IL-10 Null Mice*

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Little is known about interactions between endogenous anti-inflammatory paradigms and microvascular thrombosis in lung ischemia/reperfusion (I/R) injury. Interleukin (IL)-10 suppresses macrophage activation and down-regulates proinflammatory cytokine production, but there are no available data to suggest a link between IL-10, thrombosis, and fibrinolysis in the setting of I/R. We hypothesized that hypoxia/ischemia triggers IL-10 production, to dampen proinflammatory cytokine and adhesion receptor cascades and to restore vascular patency by fibrinolytic potentiation. Studies were performed in a mouse lung I/R model. IL-10 mRNA levels in lung were increased 43-fold over base line by 1 h of ischemia/2 h of reperfusion, with a corresponding increase in plasma IL-10. Expression was prominently localized in bronchial epithelial cells and mononuclear phagocytes. To study the link between IL-10 and fibrinolysis *in vivo*, the induction of plasminogen activator inhibitor-1 (PAI-1) was evaluated. Northern analysis demonstrated exaggerated pulmonary PAI-1 expression in IL-10 (−/−) mice after I/R, with a corresponding increase in plasma PAI/tissue-type plasminogen activator activity. *In vivo*, IL-10 (−/−) mice showed poor postischemic lung function and survival after I/R compared with IL-10 (+/+) mice. Despite a decrease in infiltration of mononuclear phagocytes in I/R lungs of IL-10 (−/−) mice, an increased intravascular pulmonary fibrin deposition was observed by immunohistochemistry and Western blotting, along with increased IL-1 expression. Recombinant IL-10 given to IL-10 (−/−) mice normalized the PAI/tissue-type plasminogen activator ratio, reduced pulmonary vascular fibrin deposition, and rescued mice from lung injury. Since recombinant hirudin (direct thrombin inhibitor) also sufficed to rescue IL-10 (−/−) mice, these data suggest a preeminent role for microvascular thrombosis in I/R lung injury. Ischemia-driven IL-10 expression confers postischemic pulmonary protection by augmenting endogenous fibrinolytic mechanisms.

Ischemia/reperfusion (I/R)[1] lung injury plays a significant role in clinical situations such as lung transplantation (1–3). Lung failure associated with I/R is characterized by increased microvascular permeability, pulmonary vascular resistance with subsequent edema formation and impairment of gas exchange, and microembolism. The lungs are particularly susceptible to ischemia/reperfusion injury, presumably due to the rich vascularity of the lungs and the relatively large surface area over which blood-borne components can interact with endothelium. The proximate mechanisms of ischemic lung injury are diverse and include leukocyte activation and recruitment (1), complement activation (4), abnormalities in pulmonary vascular tone, and increased procoagulant activity, resulting in microcirculatory failure, cellular dysfunction, edema, and cell death. The local production of proinflammatory cytokines, such as IL-1α and tumor necrosis factor-α, is considerably increased in I/R injury (5, 6), which can also feedback to increase expression of intercellular adhesion molecule (ICAM-1) or P-selectin on pulmonary vascular endothelial cells, the expression of which is likewise deleterious (7–10). Although clear roles for proinflammatory cytokines and leukocyte adhesion receptors have been defined in the setting of frank pulmonary I/R (1, 11, 12), the pathophysiological role for localized thrombosis has been ascribed only by inference.

Since microvascular thrombosis can impede the return of blood flow even when perfusion pressure is normalized, this can exacerbate and create ongoing tissue damage. In the brain, postischemic microvascular thrombosis and leukocyte recruitment contribute significantly to ischemic cerebral tissue damage (13–15). In the heart, postischemic no reflow has been documented even following relief of the major vascular obstruction. Although the lungs are a particularly vulnerable tissue in terms of their response to I/R injury, and even relatively minor interruptions of blood flow might lead to postischemic hypoperfusion and microvascular dysfunction (16), the contribution of *in situ* thrombosis to the postischemic no-reflow phenomenon in the lungs remains unclear. The important role of fibrinolysis by the plasminogen activator system has been well studied in the case of large macrovascular thrombotic occlusions, and exogenous tissue-type plasminogen activator (tPA) has been widely used in clinical settings such as acute myocardial infarction or deep vein thrombosis. Plasminogen activator inhibitor-1 (PAI-1) is a 52-kDa serine protease inhibitor that serves as the major plasma inhibitor of tPA and urokinase-type plasminogen activator (uPA) and therefore has been the focus for

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[1] The abbreviations used are: I/R, ischemia/reperfusion; ICAM-1, intercellular adhesion molecule-1; PAI-1, plasminogen activator inhibitor-1; IL, interleukin; rmIL-10, recombinant murine IL-10; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; sICAM-1, soluble intercellular adhesion molecule-1.

study as the critical inhibitor of fibrinolysis (17–22). Some studies have suggested a relation between the increased synthesis of PAI-1 and persistence or recurrence of thrombosis (17, 18) even after thrombolytic therapy. We have shown the physiologic relevance of hypoxia-induced modulation of the fibrinolytic response in the pathogenesis of fibrin accumulation in lungs using PAI-1-, tPA-, and uPA-deficient mice (23). Since hypoxia is an important component of the ischemic vascular milieu, these data suggest that I/R injury might involve not only induction of the inflammatory response but also abnormalities in the fibrinolytic system that lead to clot formation.

In most biological systems, when one set of pathways is triggered, countervailing forces are activated to modulate the effects of uncontrolled activation of the primary pathway. The current studies were undertaken to elucidate the potential negative regulatory effects of IL-10 on critically relevant issues of cytokine induction and thrombosis in lung I/R injury. IL-10 is one of the Th2 type cytokines that is believed to exert anti-inflammatory effects in different systems by its ability to suppress macrophage activation and down-regulate proinflammatory cytokine production (24). IL-10 inhibits several macrophage functions, including antigen presentation to T cells, synthesis of several proinflammatory cytokines (such as IL-1α and -β, IL-6, IL-8, tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor, and granulocyte colony-stimulating factor), and production of reactive oxygen intermediates and nitric oxide (25–27). With regard to the lung, several studies have shown that IL-10 reduces the intensity of cellular recruitment in pulmonary inflammation and is an inhibitor of the induced release of several proinflammatory cytokines such as TNF-α and macrophage inflammatory proteins 1 and 2, supporting an anti-inflammatory role of IL-10 in the lung (28). Furthermore, some studies have shown that IL-10 has significant protective effects in lung inflammatory injury by suppressing the expression ICAM-1 (29, 30). Although a recent report shows that IL-10 may inhibit coagulation and potentiate the fibrinolytic system in human endotoxemia (31), no data are available with respect to its effects on the coagulant/fibrinolytic mechanism in I/R. Therefore, the current studies were driven by a 2-fold hypothesis: 1) that microvascular thrombosis represents a significant component of lung I/R injury and 2) that endogenous IL-10 plays a pivotal role in regulating the fibrinolytic system in lung I/R injury.

EXPERIMENTAL PROCEDURES

Animals—IL-10-deficient mice (IL-10 (−/−), C57/6-IL-10^{tm1cgn}, male, 10 weeks old) (32) and their wild-type controls (IL-10 (+/+), C57/6J, male, 10 weeks old), which were purchased from Jackson Laboratories (Bar Harbor, ME), were used in these experiments according to a protocol approved by the Institutional Animal Care and Use Committee at Columbia University, in accordance with guidelines of the American Association for the Accreditation of Laboratory Animal Care.

Murine Ischemia/Reperfusion Model—Animals were initially anesthetized intraperitoneally with 0.1 mg/g of mouse weight of ketamine and 0.01 mg/g of mouse weight of xylazine, followed by intraperitoneal continuous infusion of one-third of the initial dose per hour using a syringe pump (model 100 series, KD Scientific Inc.). After ensuring appropriate depth of anesthesia, mice were intubated via tracheostomy and placed on a Harvard ventilator (tidal volume = 0.75 ml, respiratory rate = 120/min) with room air, followed by bilateral thoracotomy. The left hilum was cross-clamped for a period of 1 h, after which the cross-clamp was released. Reperfusion proceeded from 1 to 3 h according to the following groups: untreated lung in sham operation; group I, 1-h ischemia without reperfusion; and R-1, R-2, or R-3 groups, consisting of 1-h ischemia followed by 1-, 2-, and 3-h reperfusion, respectively. After observation, blood samples were obtained for ELISA (IL-10), and lung specimens were taken for Northern blot analysis.

Survival Experiments—For all experiments, the surgical operator was blinded by a colleague in the laboratory as to either the strain of mice being used (all mice were black in appearance) or to the specific substance being injected. Four groups were studied: 1) IL-10 (+/+) mice (received 300 μl of PBS without additive); 2) IL-10 (−/−) mice (received 300 μl of PBS without additive); 3) IL-10 (−/−) mice given 1 μg of recombinant murine IL-10 (R & D Systems) after thoracotomy but before pulmonary ischemia. rmIL-10 was prepared as 1 μg/300 μl in PBS; 4) IL-10 (−/−) mice given 1.0 mg/kg recombinant hirudin (direct and specific thrombin inhibitor; Sigma) after thoracotomy but before pulmonary ischemia. Recombinant hirudin was prepared for a 1.0 mg/kg injection in 300 μl in PBS. For all four groups, the experimental procedures were as follows. After 1-h ischemia followed by 2-h reperfusion, the contralateral (right) hilum was permanently ligated, so that the animal's survival and gas exchange depended solely upon the reperfused lung, and observation continued for 1 h among these four groups. As the mouse continued to be ventilated, death of the mouse was defined as a combination of 1) cessation of regular cardiac activity; 2) the apparent collapse of the left atrium; and 3) brief clonic activity indicating cessation of cerebral blood flow. At the time of death, blood samples were obtained for ELISA (IL-1α, sICAM-1) or PAI/tPA activity assays, and lung specimens were taken for the measurement of wet/dry ratios or Northern blot analyses .

In a separate series of survival experiments, lung function was ascertained by arterial blood gas analysis (sampled from the left ventricle) in mice that survived for 30 min after right hilar ligation. Immediately after determination of lung function, mice were heparinized, and lung specimens were taken for Western blot or immunohistochemical analysis for fibrin. These experiments were performed as a separate group so that obtaining the left ventricular sample of blood did not impact on mouse survival.

Wet/Dry Ratio—When mice were sacrificed after the survival experiments, the left hilum was ligated, and then the left lung (including residual blood) was taken and weighed as a wet weight. The lung specimen was desiccated at 80 °C for 24 h and weighed again as dry weight. Wet weight was divided by dry weight for the calculation of wet/dry ratio.

ELISA for IL-10, IL-1α, and sICAM-1—IL-10 (+/+) mice were divided into untreated, I, R-1, R-2, and R-3 groups. In each group, blood was drawn from the heart, kept at 4 °C overnight, and centrifuged at 13,000 rpm for 20 min to obtain serum, which was then divided into aliquots and frozen at −80 °C until the time of use. The serum IL-10 level was assayed by ELISA kits (R & D Systems), and IL-1α and sICAM-1 levels were assayed by an ELISA kit (Endogen). The lower limits of detection for IL-10, IL-1 α, and sICAM-1 assays are 4 pg/ml, 6 pg/ml, and 5 ng/ml, respectively. Values are expressed as the mean ± S.E. of duplicate determinations.

RNA Extraction from Lung Tissues and Northern Blot Analysis—In dedicated experiments, the left lung was rapidly excised and snap-frozen in liquid nitrogen until the time of mRNA extraction. After tissue homogenization using a Brinkmann Polytron homogenizer, total RNA from the lung tissues was isolated by the Tryzol method (Life Technologies, Inc.), and then poly(A) mRNA were purified using Poly(A)Ttract® mRNA Isolation Systems (Promega, Madison, WI).

To detect IL-1α, IL-10, PAI-1, and tPA transcripts, equal amounts of poly(A) mRNA (2.5 μg/lane) or total RNA (25 μg/lane) were loaded onto an 0.8% agarose gel containing 2.2 M formaldehyde for size fractionation and then transferred overnight to nylon membranes (Duralon-UV™ membranes; Strategene) with 20× SSC buffer. A murine IL-10 (1.5 kilobases; American Type Culture Collection), IL-1α (789 bp), PAI-1 (900 bp; the plasmid, containing a pBS vector and a 3014-bp insert, was generously provided by M. Cole), and tPA (800 bp; composed of a 2.5-kb insert from a pKS +/− plasmid vector (33)) cDNAs were purified using a Qiagen II gel extraction kit (QIAGEN Inc.). These fragments were used as cDNA probes after ^{32}P-random primer labeling (Prime-It RmT; Strategene) with [α-^{32}P]dCTP. After prehybridization and hybridization using QuikHyb hybridization solution (Strategene) at 68 °C for 1 h, the blots were washed twice for 15 min with 2× SSC, 0.1% SDS at room temperature, followed by one wash for 30 min with 0.1× SSC, 0.1% SDS at 60 °C. Blots were developed with X-Omat AR film exposed with an intensifying light screen at −80 °C for 3 days. Normalized absorption values were obtained by densitometry scanning (Molecular Imager® System; Bio-Rad) of cDNAs including β-actin bands.

In Situ Hybridization—In order to make RNA probes for *in situ* hybridization, the polymerase chain reaction was first performed using total RNA from the lung tissue after 1-h ischemia followed by 2-h reperfusion. Reverse transcription was performed on total RNA with oligo(dT) primers, and amplification was carried out for 35 cycles by polymerase chain reaction with specific primers for IL-10 (CLONTECH): 5′ primer, 5′-ATGCAGGACTTTAAGGGTTACTTGGGTT-3′; 3′ primer, 5′-ATTTCGGAGAGAGGTACAAACGAGGTTT-3′. An aliquot of the polymerase chain reaction product mixture was run in a 1% agarose

gel stained with ethidium bromide. The polymerase chain reaction products (455 bp) were recovered using a Qiagen II gel extraction kit (QIAGEN) and inserted to pGEM-T® Easy Vector using the T4 ligation method (Promega). The RNA expression plasmid was linearized with *Nco*I and *Sal*I enzymes to allow *in vitro* run-off synthesis of both sense- and antisense-oriented RNA probes. Both sense and antisense probes were labeled by transcription with a digoxigenin RNA labeling kit (Roche Molecular Biochemicals), and the labeled probes were then purified.

Both untreated lungs and left lungs after 1-h ischemia/2-h reperfusion were snap-frozen embedded in OCT compound (Miles Scientific) in a cryomold in liquid nitrogen. The frozen sections were cut at 5 μm thick and placed on glass slides precoated with opaque (VWR Scientific Products). Briefly, slides were prefixed in 4% paraformaldehyde for 20 min and then digested with 14 μg/ml proteinase K in Tris-EDTA (pH 8.0) for 15 min at 37 °C, fixed in 4% paraformaldehyde for 10 min. Sections were acetylated with 0.1 mol/liter triethanolamine (pH 8.0) with 0.25% (v/v) acetic anhydride. Sections were then equilibrated for 60 min in hybridization buffer consisting of 4× SSC, 50% formamide, 5% dextran sulfate, 0.1 mg/ml yeast tRNA, and 0.05 mg/ml salmon sperm DNA. Hybridization was carried out overnight at 45 °C with either IL-10 sense or antisense probe (1:25 dilution in prehybridization buffer). Sections were subjected to stringent washes consisting of a single wash with 2× SSC, two 30-min washes with 1 × SSC at room temperature, two 30-min washes with 0.1× SSC at 37 °, and two 20-min washes with Tris buffer (100 mmol/liter Tris-HCl, 150 mmol/liter NaCl). After blocking with blocking buffer (0.1% Triton X-100, 4% sheep serum, 100 mmol/liter Tris-HCl, and 150 mmol/liter NaCl), sections were incubated with a 1:100 dilution of anti-digoxigenin antibody (Roche Molecular Biochemicals) for 2 h at room temperature. After four washes, color was allowed to develop for 4 h, and development was stopped by dipping the slides briefly in Tris-EDTA buffer (pH 8.0) and then rinsing. Sections were covered with coverslips with water-soluble mounting medium.

PAI and tPA Activity Assay—PAI/tPA activity was determined by a functional rate assay described by Ranby *et al.* (34) and its adaptation to plasma samples, as described by Wiman *et al.* (35). Blood samples (F, $n = 9$; IL-10 (+/+), $n = 9$; IL-10 (−/−), $n = 9$; and IL-10 (−/−) plus rmIL-10, $n = 9$) were drawn at the end of survival experiments and acidified by acetate buffer immediately. The samples were centrifuged at 2000 × *g* for 5 min. Equal volumes of acetate buffer and Tris buffer were added to acidified plasma and incubated at 37 °C for 20 min. The activity was assayed by Spectrolyse® tPA/PAI activity assay kits (American Diagnostica). In brief, each sample was added to reaction mixture containing a known quantity of tPA, soluble fibrin (Desafib; American Diagnostica), and a plasmin substrate (Spectrozyme PL; American Diagnostica). Plasmin generated by the reaction of tPA and fibrin cleaves the Spectrozyme substrate to generate a yellow color, which can be measured at an OD of 405 nm. PAI activity is expressed as the amount of PAI that inhibits 1 IU of tPA.

Western Blotting-for Fibrin-Accumulation—Lung tissues were harvested following systemic heparinization and snap-frozen in liquid nitrogen until the time of fibrin extraction. These tissues were placed in buffer (0.05 M Tris, 0.15 M NaCl, 500 units/ml heparin, final pH 7.6) on ice and homogenized. Plasmin digestion was performed by a modification of the methods of Francis (36), as described previously (34). Human plasmin (0.32 units/ml; Sigma) was added to the tissue homogenate, followed by agitation at 37 °C for 6 h. More plasmin (0.32 units/ml) was then added, and samples were agitated for an additional 2 h, and then the mixture was centrifuged at 2300 × *g* for 15 min, and the supernatant was aspirated. As a positive control, mouse fibrinogen (2.5 mg in 0.25 ml; Sigma) was clotted with human thrombin (4 units; Sigma) in Tris-buffered saline (1.75 ml) in the presence of calcium chloride (0.013 ml of 2.5 M) for 4 h at room temperature. Clotted fibrinogen was centrifuged for 5 min, and the pellet was suspended in Tris-buffered saline (1.0 ml) containing human plasmin (0.32 units/ml) and agitated at 37 °C. Additional plasmin (0.32 unit/ml) was added after 6 h, and samples were agitated for an additional 2 h. As a negative control, unclotted mouse fibrinogen was processed in an identical manner. Protein concentration of plasmin-treated lung supernatants and plasmin-treated unclotted and clotted fibrinogen solutions was measured by the Bradford method (37) before loading the gel. Samples were boiled for 3 min under reducing conditions, loaded onto a SDS-polyacrylamide gel (7.5% reduced gel; 10 μg of protein/lane), and subjected to electrophoresis. Samples were electrophoretically transferred to nitrocellulose, and blots were reacted with a monoclonal anti-fibrin IgG1 (Biodesign International) that had been prepared with human fibrin-like β peptide as immunogen (38). The cross-reactivity of this antibody with murine fibrin was confirmed by blotting with the positive (murine fibrin) and negative (murine fibrinogen) controls prepared as described above. Secondary detection of sites of primary antibody localization was accomplished using a horseradish peroxidase-conjugated goat anti-mouse IgG (Fc) (Sigma). Final detection of bands was performed using the enhanced chemiluminescence Western blotting system (Amersham Pharmacia Biotech).

Immunohistochemistry—In addition to Western blot analyses performed as described above, fibrin accumulation was determined by immunohistochemistry. Left lung tissue from untreated and IL-10 (−/−) groups, harvested in survival experiments (with antemortem heparinization to limit postmortem thrombosis) was used to identify the fibrin accumulation by immunostaining. The left lung was snap-frozen embedded in OCT compound, and sections were cut at 5 μm thick, air-dried, and acetone-fixed. Endogenous peroxidase activity was blocked by incubation for 20 min in PBS containing 0.3% hydrogen peroxide. Sections were immunostained using the same primary antibody (1:50) as that used for Western blotting, which is reactive to murine fibrin. Sites of primary antibody binding were visualized with mouse ExtraAvidin® alkaline phosphatase staining kit (Sigma) and Sigma FAST 228 FAST RED (Sigma). In order to more specifically localize fibrin deposits, a double immunostaining technique was employed on these same sections. Sections were overlaid with 20% goat serum for 30 min, washed, and then incubated for 1 h at room temperature with a rabbit polyclonal antihuman von Willebrand's antibody (Cortex Biochem, San Leandro CA). Detection of the primary antibody was accomplished using a biotinylated goat anti-rabbit IgG and the peroxidase avidin-biotin staining procedure. Immunostaining for mononuclear phagocytes was accomplished using a primary rat monoclonal anti-mouse panmacrophage marker (MOMA-2; BIOSOURCE International, Camarillo, CA) (39). Development and visualization were accomplished as described above with the exception that slides were counterstained with methyl green. The number of positively stained macrophages was determined in 10 random high power fields (× 400 magnification), and the average number of macrophages/field was calculated for each group.

Statistical Analysis—The data were expressed as mean ± S.E. All statistical comparisons were performed using a commercially available statistical package for the Macintosh personal computer (STAT VIEW-J 5.0; Abacus Concepts). Analysis of variance was used to compare different conditions among the groups of mice. The product limit (Kaplan-Meier) estimate of the cumulative survival was assessed with the log-rank test to evaluate significance differences. Differences were considered significant at the level of $p < 0.05$.

RESULTS

ELISA for IL-10, IL-1α, and sICAM-1—To investigate the role of IL-10 in lung I/R injury, we first examined the serum levels of IL-10, IL-1α, and sICAM-1 in both IL-10 (+/+) and (−/−) mice during ischemia and reperfusion. Serum levels of IL-10 in IL-10 (+/+) mice increased time-dependently (R-3 was the longest time studied) (Fig. 1*A*). Under conditions of 1 h ischemia/2 h reperfusion, IL-1α levels in IL-10 (−/−) mice were significantly higher than those in IL-10 (+/+) mice. Administration of rmIL-10 to IL-10 (−/−) mice reduced levels of IL-1α significantly (Fig. 1*B*). No significant differences in sICAM-1 levels were noted among these four groups (Fig. 1*C*).

Time Course of IL-10 and IL-1α mRNA Expression in Mouse Lung I/R—To investigate the time course of proinflammatory cytokine IL-1α and anti-inflammatory cytokine IL-10 expression in our model, 2.5 μg of poly(A) RNA was derived from the untreated, I, R-1, and R-2 groups (five lungs were homogenized for each group to isolate 2.5 μg of poly(A) RNA for each group). mRNA was loaded into each lane of an agarose gel, and Northern blotting procedures were performed as described. IL-1α mRNA expression was up-regulated as early as 1 h after ischemia, and this increase continued after reperfusion (7.1-fold increase by 2 h of reperfusion) (Fig. 2, *A–C*). Although IL-10 mRNA induction during ischemia was modest, induction during reperfusion was even more pronounced (43-fold increase at 2 h of reperfusion).

Localization of IL-10 mRNA Expression in I/R Lungs—To localize the cells in the lungs in which IL-10 mRNA was in-

Exhibit 4

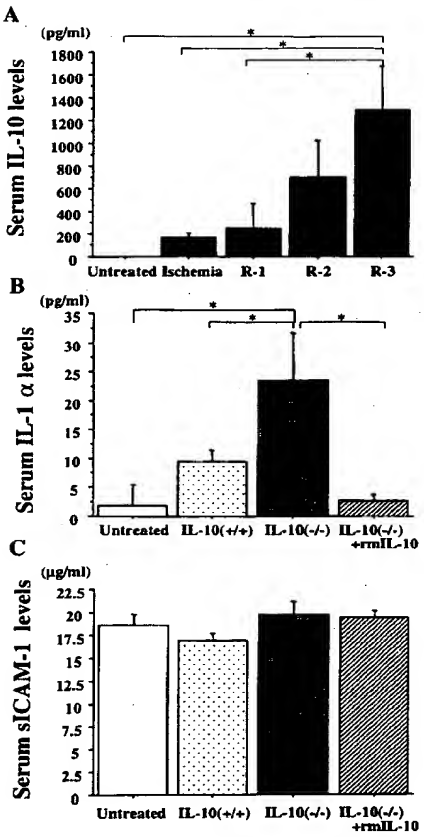

FIG. 1. **Serum levels of IL-10, IL-1 α, and sICAM-1 were measured by ELISA.** IL-10 in the I/R model showed a time-dependent increase after reperfusion (*A*). For IL-1 α and sICAM-1, the values were compared among untreated, IL-10 (+/+), (−/−), and (−/−) plus rmIL-10 mice. IL-1α levels in the IL-10 (−/−) group showed elevated values compared with other groups, which were reduced by exogenous rmIL-10 administration (*B*). There was no significant difference between sICAM-1 levels among these groups (*C*). Means ± S.E. are shown. *, $p < 0.05$.

duced by I/R, *in situ* hybridization was performed using murine sense-and-antisense probes. Lung tissue from the R-2 group demonstrated increased IL-10 mRNA levels in bronchial epithelial cells (Fig. 3*A*) and mononuclear cells (Fig. 3*C*), but not in the endothelial cells. As negative controls, this staining was not observed in antisense-stained adjacent sections (Fig. 3, *B* and *D*) or in untreated lungs (Fig. 3, *E–H*). Quantitative analysis of these data indicates a 17-fold increase in IL-10 mRNA under ischemic compared with untreated control conditions for epithelium and an 11-fold increase for mononuclear phagocytes.

PAI-1 mRNA Expression in IL-10 (+/+), (−/−), and (−/−) Plus rmIL-10 Mice Lungs—To investigate the contribution of IL-10 to the fibrinolytic balance in I/R, PAI-1 mRNA expression was studied by Northern blot analysis in IL-10 (+/+), IL-10 (−/−), and IL-10 (−/−) plus rmIL-10 mice. Blots were performed four separate times using four mice in each group, and normalized absorption values (by densitometry scanning) were analyzed statistically. One-h ischemia/2 h reperfusion up-regulated PAI-1 mRNA levels compared with untreated lung in IL-10 (+/+) mouse (2.6-fold increase) (Fig. 4, *A* and *B*). PAI-1 expression was significantly up-regulated in IL-10 (−/−) mice (4.7-fold increase); this up-regulation was suppressed by administration of exogenous rmIL-10 (Fig. 4, *A* and *B*). In contrast to increased PAI-1 mRNA expression, although tPA

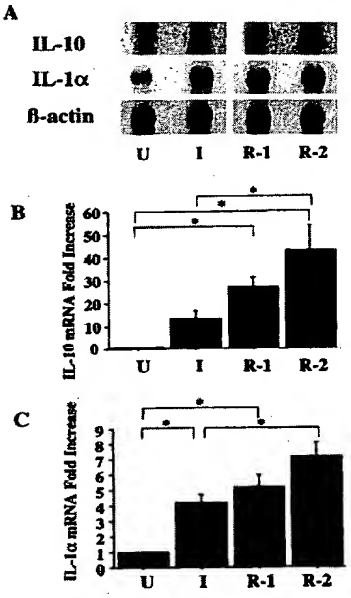

FIG. 2. **Time course of IL-10 and IL-1α mRNA expression after I/R in IL-10 (+/+) mice lungs.** Northern blots to investigate the time course during 1-h ischemia followed by a 2-h reperfusion period in this mouse I/R model. Blots were stripped and then probed with a cDNA for β-actin in order to confirm equal loading of lanes (*A*). Densitometric scanning analysis is shown for IL-10 mRNA (*B*) and IL-1 α mRNA expression (*C*).

mRNA appeared to increase in IL-10 (−/−) mice, this difference did not achieve the level of a significant difference on multiple blots (data not shown). These data suggested that in wild-type mice, IL-10 induction might contribute to up-regulation of fibrinolytic activity in lung I/R injury.

PAI and tPA Activity Assay—Because PAI can circulate in both active and latent forms (40), plasma PAI activity was measured using a microtiter system that monitors PAI-mediated inhibition of plasminogen activator activity (34, 35) as well as tPA activity. IL-10 (−/−) mice showed significantly higher PAI activity compared with that of IL-10 (+/+) mice. PAI activity was significantly reduced by administration of exogenous rmIL-10 (Fig. 5*A*). Although lack of the IL-10 gene did not appear to alter tPA activity, reconstitution of the IL-10 null mice with rmIL-10 appeared to augment tPA activity (Fig. 5*B*).

Although tPA mRNA is reportedly unchanged after endothelial exposure to anoxia (41) or hypoxia (42), the PAI/tPA activity ratio appears to be increased (42), which may contribute to the apparent hypofibrinolytic state of endothelial cells exposed to hypoxia *in vitro* (42). In a whole animal hypoxia model, it appears that tPA mRNA levels are actually reduced in the lungs, which, along with induction of PAI-1 mRNA, may provide a potent stimulus for thrombus accrual (23). In the current lung I/R model, the PAI/tPA ratio was therefore calculated to provide insights into the relative fibrinolytic "balance" in this model. The PAI/tPA ratio in IL-10 (−/−) mice was significantly greater than that observed in IL-10 (+/+) mice, and this ratio was normalized by reconstitution of IL-10 null mice with rmIL-10 (Fig. 5*C*).

Detection of Fibrin—The data shown so far regarding the role of IL-10 in modulating the fibrinolytic state suggest that *in vivo*, changes in the fibrinolytic balance in IL-10 (−/−) mice incited by I/R are likely to be of pathologic significance with respect to the accrual fibrin. Immunohistochemical analysis revealed that I/R-driven fibrin accumulation occurred predom-

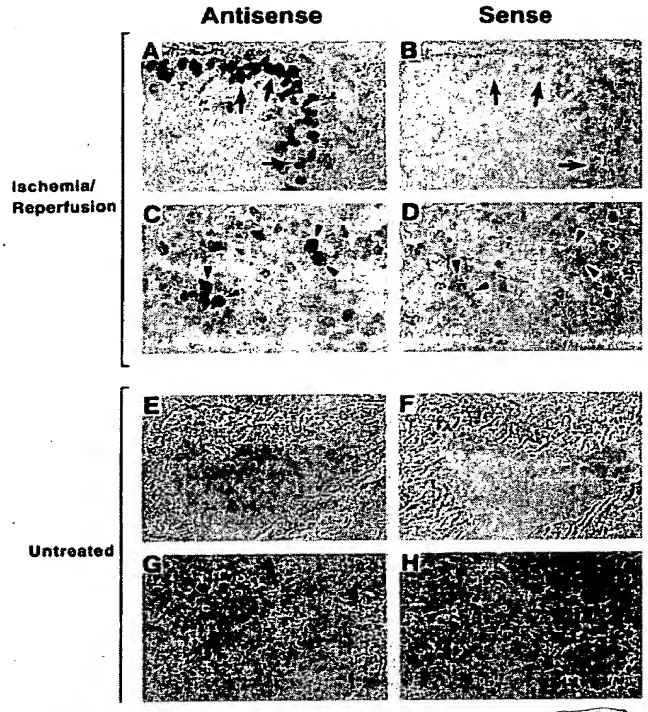

FIG. 3. **Localization of mRNA for IL-10.** Serial sections of lung tissue were taken from 1-h ischemia, followed by 2-h reperfusion group (*A–D*) and lungs from untreated mice (*E–H*). *In situ* hybridization for IL-10 mRNA expression was performed using either a murine sense probe (*B*, *D*, *F*, and *H*) as a negative control or an antisense probe (*A*, *C*, *E*, and *G*) to detect the presence of IL-10 mRNA. IL-10 mRNA was identified in the bronchial epithelial cells (*arrows* in *A*) as *blue-purple* coloring. Mononuclear cells also showed prominent expression (*arrowheads* in *C*; note that these represent adjacent sections).

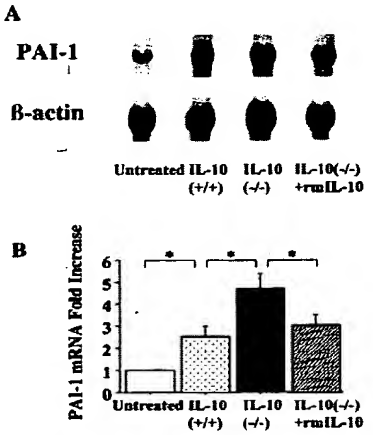

FIG. 4. **PAI-1 mRNA expression in untreated, IL-10 (+/+), (−/−), and (−/−) plus rmIL-10 mice lungs (*A*) and quantitative analysis by densitometric scanning (*B*).** Normalized absorption values were obtained by densitometric scanning of PAI-1 and β-actin bands. Data are shown as means ± S.E. *, $p < 0.05$.

inantly at intravascular sites (Fig. 6*A*); controls showed a relative absence of fibrin accumulation in untreated lung sections stained with identical procedures or in I/R lung tissue subjected to similar staining procedures in the absence of the primary anti-fibrin antibody (Fig. 6*B*). To confirm that IL-10 (−/−) mice actually exhibit I/R-induced accumulation of fibrin,

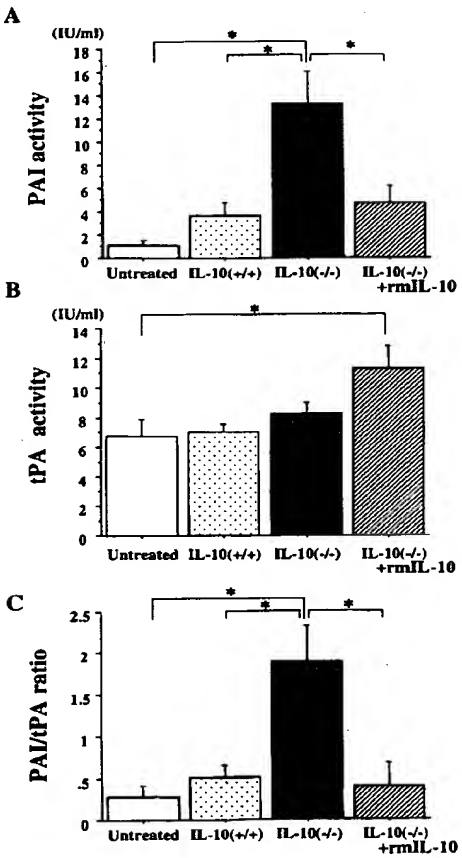

FIG. 5. **Effect of endogenous IL-10 on PAI and tPA activity.** All blood samples were drawn at the end of survival experiments and acidified by acetate buffer immediately ($n = 9$ in each group). *A*, PAI activity; *B*, tPA activity; *C*, PAI/tPA ratio. This ratio was calculated as PAI activity/tPA values in each group. The values expressed are the means ± S.E. *, $p < 0.05$.

fibrin accumulation was quantified using two different methods in tissue from mice heparinized immediately prior to sacrifice to reduce nonspecific/postmortem thrombosis. In the first method, vessels staining for fibrin were counted by an observer blinded to experimental conditions (Fig. 6*C*). Although I/R increased the number of fibrin-positive vessels significantly, there was an even more marked increase in fibrin-positive vessels in the IL-10 (−/−) mice. Recombinant murine IL-10 reduced the number of fibrin-positive vessels, suggesting a direct role of IL-10 in fibrin accumulation following I/R. In the second method for quantifying fibrin accumulation, immunoblotting for fibrin was performed on lung tissue. IL-10 (+/+) mice showed that the I/R stimulus does indeed cause fibrin accumulation, compared with the absence of detectable fibrin in untreated lung (Fig. 6*D*). IL-10 (−/−) mice showed a marked increase in fibrin accumulation compared with that seen under identical I/R conditions in IL-10 (+/+) mice. Note that IL-10 (−/−) mice given hirudin (1.0 mg/kg) also had a marked diminution in I/R-induced fibrin accumulation. Provision of exogenous rmIL-10 to reconstitute the IL-10 null mice resulted in marked suppression of fibrin accumulation in lung tissue (Fig. 6, *C* and *D*). These data demonstrate that endogenous IL-10 plays a pivotal role in potentiating fibrinolysis and reducing fibrin accumulation after I/R injury.

Quantification of Leukocyte Infiltration—In order to deter-

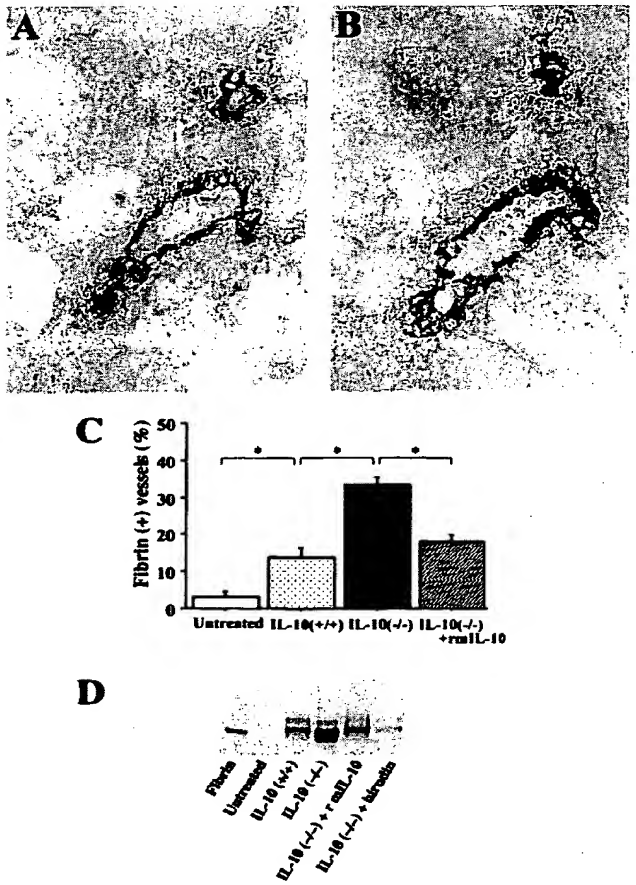

FIG. 6. **Effect of endogenous IL-10 on I/R-induced fibrin accumulation as detected by immunohistochemistry and immunoblotting for fibrin (with antemortem heparinization to limit postmortem thrombosis).** *A*, fibrin (red staining) is seen to have accumulated in the ischemic/reperfused vessels (delineated by the endothelial marker, von Willebrand's; *black*) in *A*. *B* represents an adjacent section stained in the absence of the primary anti-fibrin antibody. *C*, quantification of intravascular fibrin accumulation determined by counting fibrin-positive vessels per high power field. *D*, plasmin digests of lung tissue taken from the following groups were used as an additional way to quantify fibrin deposition: fibrin (prepared from clotted fibrinogen *in vitro* as a positive control), untreated, IL-10 (+/+), IL-10 (−/−), IL-10 (−/−) plus rmIL-10, and IL-10 (−/−) plus hirudin mice (these conditions are as described under "Experimental Procedures").

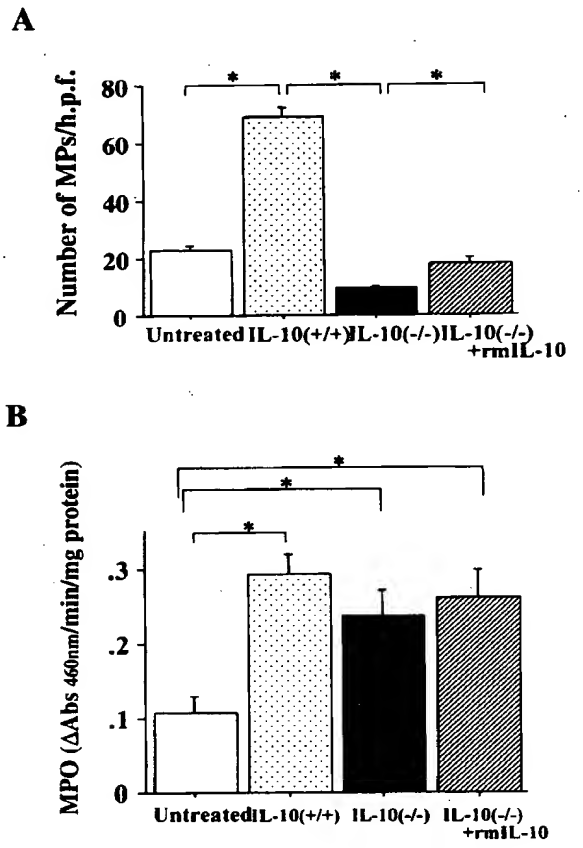

FIG. 7. **Quantification of leukocyte infiltration.** Specific immunostaining for macrophages (*A*) or myeloperoxidase assays (*B*) was performed to quantify polymorphonuclear leukocyte infiltration. Experimental conditions were identical to those shown in Fig. 4.

mine whether IL-10 modulates the recruitment of leukocytes (mononuclear phagocytes or polymorphonuclear leukocytes) in the setting of lung I/R injury, specific immunostaining and myeloperoxidase assays were performed. These data show that IL-10 (+/+) mice demonstrated increased recruitment of both leukocyte types following lung I/R injury (Fig. 7, *A* and *B*). Mice in which the IL-10 gene was absent exhibited reduced accumulation of both leukocyte types, but particularly of mononuclear phagocytes. Reconstitution of IL-10 null mice with rmIL-10 resulted in an intermediate level of accumulation.

Arterial Blood Gas Analysis—Because these data show pathological accumulation of fibrin in I/R and especially in IL-10 (−/−) mice exposed to I/R, additional experiments were performed to show that the pathological accumulation of fibrin is liable to be pathologically relevant. Arterial blood samples were taken 30 min after 1-h ischemia/2-h reperfusion from IL-10 (+/+) mice, IL-10 (−/−) mice, rmIL-10-reconstituted IL-10 (−/−) mice, and IL-10 (−/−) mice given hirudin. For these experiments, the contralateral (nonischemic right) lung was excluded from the circulation so that both animal survival and gas exchange were completely dependent upon the function of the postischemic left lung. Arterial oxygenation and PaO_2 deteriorated in IL-10 (−/−) mice compared with IL-10 (+/+) mice, while exogenous rmIL-10 significantly ameliorated these hallmarks of lung function. IL-10 (−/−) given hirudin mice also showed significant improvement in PaO_2 compared with IL-10 (−/−) mice (Fig. 8*A*). $PaCO_2$ tracked the arterial oxygenation data in inverse relationship, as one would expect (Fig. 8*B*).

Wet/Dry Ratio—To further assess lung tissue damage after I/R, we measured wet/dry ratio after the completion of the survival experiments. The data showed that IL-10 (−/−) mice contained significantly more water than did IL-10 (+/+) mice, while edema formation was reduced by the administration of rmIL-10 or hirudin (Fig. 8*C*).

Survival—Because *in vivo*, there are many different mechanisms contributing to lung injury and demise of an animal after an ischemic insult, survival experiments were performed to "summate" the multitude of competing forces and to establish the role of endogenous IL-10 and thrombosis in lung I/R injury. Again for these experiments, following ischemia and reperfusion, the contralateral (nonischemic right) lung was excluded from the circulation so that survival depended entirely on the postischemic left lung. IL-10 (+/+) mice subjected to 1 h of ischemia followed by 2 h of reperfusion showed 67% survival

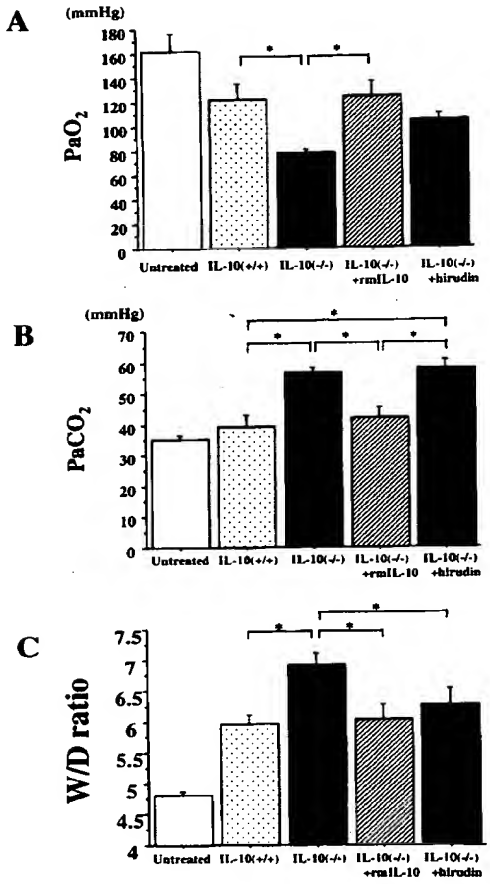

FIG. 8. **Effect of endogenous IL-10 on lung function after 1 h of ischemia followed by 2 h of reperfusion.** *A* and *B*, effect on gas exchange. Arterial blood samples were drawn from untreated and IL-10 (+/+), IL-10 (−/−), IL-10 (−/−) plus rmIL-10, and IL-10 (−/−) plus hirudin mice that survived for 30 min after right hilar ligation ($n = 7$ in each group). *C*, effect on edema formation, measured as the wet/dry weight ratio of the excised lung tissue. Wet/dry ratio was calculated among five groups, including untreated lung ($n = 9$ in each group). The values expressed are the means ± S.E. *, $p < 0.05$.

during 60 min of observation after ligation of the right hilum. Survival was significantly less in IL-10 (−/−) (11%) mice during the same observation period. Reconstitution of IL-10 null mice with exogenous rmIL-10 improved not only lung function but also the survival (44%) significantly. To demonstrate that thrombus accumulation is a critical mechanism responsible for the poor survival of IL-10 null mice after lung I/R, a direct and specific thrombin inhibitor, recombinant hirudin was administered to IL-10 (−/−) mice prior to ischemia. One mg/kg of recombinant hirudin markedly improved survival of IL-10 (−/−) mice (78%). These data suggest that thrombus accumulation is a significant cause of high mortality in IL-10 (−/−) mice (Fig. 9).

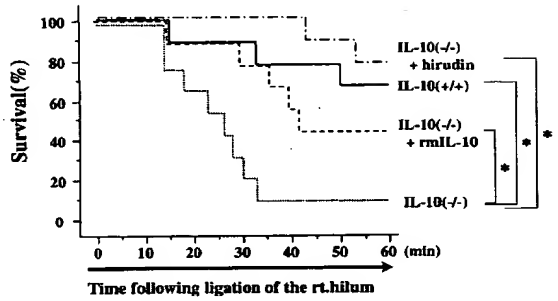

FIG. 9. **Effect of endogenous IL-10 on mouse survival after I/R injury.** Animal survival depended solely upon the ischemia/reperfused lung (the contralateral lung was excluded from the circulation; mice were observed for 1 h after exclusion of the contralateral lung). IL-10 (+/+), IL-10 (−/−), IL-10 (−/−) plus rmIL-10, and IL-10 (−/−) plus recombinant hirudin ($n = 9$ in each group) mice were used. The product limit (Kaplan-Meier) estimate of the cumulative survival was assessed with the log-rank test to evaluate for significant differences in survival. *, $p < 0.05$.

DISCUSSION

The major findings in these experiments are as follows: 1) endogenous IL-10 expression increases following lung ischemia and reperfusion in parallel with IL-1α expression; 2) the absence of the IL-10 gene increases PAI-1 expression and results in augmented fibrin accumulation in postischemic lungs; 3) thrombus accumulation is a significant adverse event responsible for poor postischemic lung function and survival; and 4) provision of recombinant IL-10 (or an anti-thrombin agent) can rescue IL-10 null mice from thrombus accumulation and lung failure following ischemia. Since IL-10 null mice exhibited reduced recruitment of mononuclear phagocytes but the highest levels of fibrin, the potentiation of fibrinolysis by IL-10 cannot be explained on the basis of IL-10 suppressing mononuclear phagocyte infiltration into the lungs following I/R. More likely, IL-10 has a direct effect on macrophages to reduce PAI-1 expression, a claim that is indirectly supported by *in vitro* data (not shown) in which mononuclear phagocytes exhibited IL-10-mediated suppression of hypoxic induction of PAI-1. *In vivo*, IL-10 not only potentiates fibrinolysis but suppresses the expression of a potent proinflammatory cytokine (IL-1α), whose expression in ischemia also contributes to leukocyte recruitment and tissue damage (43).

Like many other cytokines, IL-10 is produced by many cell types and mediates diverse cellular functions. In addition to T cells, IL-10 is also expressed by stimulated B lymphocytes, monocytes-macrophages (44), keratinocytes (45, 46), mast cells (46), and epithelial cells (47). With regard to the lung, it has been demonstrated that alveolar macrophages can produce significant amounts of IL-10 (48). In our study, *in situ* hybridization identified mononuclear phagocytes and bronchial epithelial cells as major cellular sources of IL-10 in lung. Bonfield *et al.* (49) have demonstrated that bronchial epithelial cells from healthy control subjects constitutively produce IL-10, which appears to be down-regulated in cystic fibrosis patients. However, in our acute lung I/R model, Northern blot analysis and serum levels showed that IL-10 was expressed little constitutively but strongly up-regulated in a time-dependent fashion after reperfusion; this may serve as compensatory regulation against the inflammatory response after ischemic injury.

The prevailing belief is that the mechanism by which IL-10 exerts cytoprotective effects against I/R injury is due to 1) the ability of IL-10 to inhibit macrophage function and to inhibit the synthesis of several proinflammatory cytokines (25–28) and 2) its ability to suppress leukocyte-endothelial cell interactions (29, 30). In order to elucidate the role of endogenous IL-10 in lung I/R injury, we used IL-10-deficient mice, which could be reconstituted with exogenous rmIL-10, as a critical way to dissect the mechanism by which IL-10 works *in vivo*. According to our data, IL-10 (−/−) mice showed the greatest expression of IL-1α, prominent edema formation, the worst postischemic lung function, and the lowest survival compared with IL-10 (+/+) mice. Whereas exogenous rmIL-10 administration reversed these adverse effects (including the high mortality),

sICAM-1 levels were not significantly affected by the IL-10 deficiency (nor was ICAM-1 on Northern blots; data not shown). These data suggest that although endogenous IL-10 suppresses IL-1 expression, its protective role in lung ischemia is not likely to be mediated by inhibiting ICAM-1 expression.

In contrast to our initial expectations that macrophage infiltration might be suppressed by IL-10, our data showed exactly the opposite effect, that the presence of IL-10 was associated with increased accumulation of mononuclear phagocytes. Similar albeit much less pronounced effects were seen with regard to neutrophil infiltration when tissue was analyzed for the relatively neutrophil-specific enzyme myeloperoxidase. Although the I/R procedure caused a dramatic increase in the number of infiltrating MPs, lack of the IL-10 gene was associated with a significant reduction in MP recruitment. "Rescue" of the IL-10 null mice with IL-10 caused a significant increase in MP recruitment following I/R, albeit absolute levels were highest in mice capable of expressing the IL-10 gene. Although IL-10 generally has anti-inflammatory properties, there is support in the literature for an effect of IL-10 to increase levels of monocyte chemoattractant protein-1 under certain conditions (dependent upon cell type and activation state) (50).

Recent evidence is emerging that, in concert with the shift toward a procoagulant phenotype, endothelial cells exhibit a diminished fibrinolytic response under conditions of oxygen deprivation, especially when followed by reoxygenation and attendant production of reactive oxygen intermediates (41, 51). We have shown the physiologic relevance of hypoxia-induced modulation of the fibrinolytic response in the pathogenesis of fibrin accumulation using PAI-1-, tPA-, and uPA-deficient mice (23). Since microvascular thrombosis can impede the return of blood flow even after recanalization of a major vascular territory, alterations in the fibrinolytic balance can exacerbate ongoing tissue damage and edema formation. Postischemic noreflow generally consists of multiple effector mechanisms such as neutrophil plugging with enhanced adhesion receptor expression (52, 53) and microvascular thrombosis. However, our studies point to the protective role of IL-10 likely to be predominantly due to its effects on fibrin accumulation rather than leukocyte adhesion, since ICAM-1 expression was not significantly enhanced in IL-10 null mice compared with IL-10 (+/+) mice, leukocyte accumulation was actually less in the IL-10 null mice, and an anti-thrombin agent alone sufficed to normalize the postischemic pulmonary function of IL-10 null mice. Pajkrt *et al.* (31) have shown that IL-10 not only inhibits activation of coagulation, but IL-10 also modulates the fibrinolytic system (reduced tPA plasmin-α_2-anti-plasmin complexes and D-dimer) during human endotoxemia. The current studies provide the first solid evidence linking a deficiency in IL-10 to the inhibition of fibrinolytic mechanisms, fibrin accrual, and tissue injury following ischemia.

In this study, we have focused primarily on the effect of IL-10 on the regulation of the fibrinolytic system. *In vitro*, mouse macrophages subjected to hypoxia overexpressed PAI-1 mRNA, the induction of which was suppressed by exogenous rmIL-10 (data not shown). Although in pathophysiological conditions, IL-1α released from activated macrophages might stimulate themselves by an autocrine loop to induce PAI-1 expression, it remains unclear whether IL-1α expression is a necessary intermediary in the augmented PAI-1 expression seen following lung ischemia. Although IL-1α might itself promote apoptotic cell death or inflammatory mechanisms of tissue injury, the ability of recombinant hirudin to rescue IL-10 null mice argues against the IL-1 production being critically deleterious. On the other hand, inflammatory and activation pathways *in vivo* are intertwined in complex fashion, and it is remotely possible that hirudin could exert protective effects that are anti-inflammatory-based (thrombin can activate endothelial cells independent of its effects on coagulation). Notwithstanding the difficulties of precisely identifying all pathways involved in *in vivo* lung I/R injury, the preponderance of the data here suggest that the accrual of thrombus has a pivotal pathological role. The present results are the first to elucidate that ischemia-driven endogenous IL-10 might have not only anti-inflammatory effects but also regulatory effects on the fibrinolytic system that contribute to the mitigation of postischemic lung tissue injury.

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